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APOPTOSIS INDUCED BY MICROTUBULE DISRUPTING DRUGS IN NORMAL MURINE THYMOCYTES *IN VITRO*

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Abstract

Disruption of cytoplasmic and spindle microtubules by colchicine or nocodazole increases mitotic index, but it also enhances apoptosis in isolated mouse thymocytes; the apoptotic index exceeds 20% after 4 hours of incubation with either drug (5% in controls). Apoptosis was confirmed by DNA fragmentation, and was blocked by calcium chelators and inhibitors of protein synthesis. The apoptotic effect of microtubule disrupting drugs (MDD) was directed to interphase thymocytes and was independent on MDD action on mitotic cells. However, cell death of mitotically arrested cells showed ultrastructural changes similar in many aspects to apoptosis.

Key Words: Apoptosis, programmed cell death, microtubules, colchicine, nocodazole, thymocytes, mitosis, metaphase arrest, mouse, DNA fragmentation.

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Introduction

Microtubule disrupting drugs (MDD) are used in the treatment of human cancers, although the exact mechanism of their action has not been clearly established. It is generally accepted that the arrest of cells in metaphase is the dominant effect of these drugs [1], but it has also been shown that they enhance apoptosis in normal and malignant cells [5, 6, 7, 13, 14, 17, 26].

Apoptosis, a form of programmed cell death, occurs during embryological development, physiological renewal of cells, and in certain pathological states [for recent reviews, see 27 and 28]. This morphologically and biochemically distinct mode of cell death can be induced in normal and neoplastic cells by drugs, toxins, mild hyperthermia, or irradiation [4, 19, 23, 25, 36]. Despite numerous studies, the mechanisms involved in activation of the apoptotic process are not well understood. One of the biochemical events associated with apoptosis appears to be a cleavage of DNA into oligonucleosomal fragments by the endonuclease [2, 32]. It has been suggested that this DNA cleavage accounts for the appearance of distinct nuclear morphological changes [35], although they can occur without concomitant DNA fragmentation in certain cell types [12]. These changes precede fragmentation of cells into smaller membrane-bound apoptotic bodies. At the same time, ultrastructure of cytoplasmic organelles appears normal, and plasmalemma and endomembranes are intact [33, 34].

The apoptotic process, depending on the cell type, may or may not require RNA and protein synthesis [22], and an early sustained increase of calcium ions occurs in the majority of cells which undergo programmed cell death [20, 35]. However, controversy exists regarding the exact role of a particular biochemical process in apoptosis [for details, see 22 and 28].

In this study, we investigated ultrastructural characteristics of cell death induced by MDD in isolated murine thymocytes. Isolated thymocytes are often used for the study of apoptosis, and a variety of stimuli can activate this unique "cell death program" [3, 19, 24, 35, 36].

The present study demonstrates that microtubule disrupting drugs, colchicine and nocodazole, induce apoptosis in isolated murine thymocytes. This process is rapid, requires extracellular calcium ions (Ca^{2+}) and protein synthesis, and is associated with internucleosomal cleavage of DNA. In addition, cells arrested in prometaphase show degenerative changes similar in many aspects to apoptosis.

Material and Methods

Materials

Nocodazole was from Fluka (Buchs, Switzerland) and colchicine from Sigma (Deisenhofen, FR Germany). Other chemicals were from Merck (Darmstadt, FR Germany) and of the highest purity available.

Cell preparation and culture

Male C57BL mice, 4-5 weeks old (Institute for Experimental Medicine, VMA, Belgrade) were used in these experiments. Suspensions of thymocytes were obtained by mincing the glands (typically obtained from 10-15 mice) in Parker's medium on ice (Torlak, Belgrade; pH 7.4), supplemented with 5% fetal calf serum, 60 μg/ml penicillin and 100 μg/ml streptomycin, followed by passage through a 110-μm nylon mesh. More than 95% of the cells excluded Trypan blue, thus indicating that this cell isolation procedure did not impair cell viability and provided highly enriched (97.5% Thy1.1+) thymocyte suspension. Cell suspensions were diluted to a final concentration of 1-2 x 10^7 cells/ml in the same medium and placed in tissue culture flasks (5 ml per flask). When necessary, chemicals, dissolved in medium and with pH adjusted to 7.4, were added to the cell suspensions at the onset of experiment. Nocodazole was initially dissolved in dimethylsulfoximine (DMSO), and diluted to the required concentration in medium. DMSO alone, in the final concentration of 1%, did not appear to have any effect on thymocytes incubated under the conditions described.

Thymocytes were incubated for up to 4 hours at 37°C in a humidified atmosphere of 5% CO_2 in air, with or without 2 μM colchicine or 2.5 μM nocodazole. Endonuclease activity was inhibited by the addition of $ZnSO_4$ (final concentration 5 mM). Extracellular calcium was chelated by 5.0 mM of ethyleneglycol-bis-(b-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) in the medium, and protein synthesis was inhibited by the addition of cycloheximide (50 μM).

Morphological studies

Following the various treatments, cells were washed twice in phosphate buffered saline (PBS; pH 7.4) using low speed centrifugation (150 *g*, 10 minutes), and pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After osmification, pellets were stained *en block* with uranyl acetate, dehydrated in a series of graded ethanol, cleared in propylene oxide, and embedded in Epon. Semi-thin sections were stained with toluidine blue for light microscopy. Ultrathin sections mounted on copper grids were stained with lead citrate and examined in a Philips EM 200 or EM 300 electron microscope.

Quantification of apoptosis

Apoptotic, mitotic, and necrotic cells were counted on 5 randomly chosen high power fields (100x oil immersion objective) on toluidine blue stained semithin sections (one section per specimen, and 3-5 specimens per individual test), by the aid of a square eye piece graticule. The identification of apoptosis and necrosis was based on previously defined morphological criteria [31]. Briefly, apoptotic cells were characterized by cell shrinkage and condensed hyperchromatic nucleus showing nodular or crescent-like condensation of nuclear chromatin, while cells displaying alterations ranging from marked swelling to the featureless cell masses were considered to be necrotic. The percentage of apoptotic and mitotic cells was determined on an average of 1,100 cells counted per section, and expressed as apoptotic index (AI) and mitotic index (MI).

DNA extraction and electrophoresis

Fragmentation of DNA was determined as previously described [18]. Briefly, after washing cells two times in PBS, pellets were resuspended at 2 x 10^7 cells/ml in a cell lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8, supplemented with 0.5% (w/v) N-lauroylsarcosine and 0.5 mg/ml proteinase K) and incubated for 1 hour at 50°C in a water-bath. Incubation was continued for an additional hour after the addition of 0.25 mg/ml heat-treated RNAse A. Cells were extracted twice with phenol, followed by two changes of chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated with sodium acetate and ethanol overnight at -20°C, pelleted by centrifugation and resuspended in 100 μl Tris-EDTA buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8).

DNA (3 μg/per lane) was electrophoresed in 1.5% agarose gel, and photographed under ultraviolet light after staining with ethidium bromide.

Results

Morphological findings

Exposure of thymocytes to either colchicine or nocodazole causes an increase in both apoptotic and mitotic indexes (Fig. 1). The increase in MI is a consequence of the mitotic blockade due to the disruption of microtubules. The disruption of cytoplasmic and spindle microtubules (Fig. 2) seen by electron microscopy

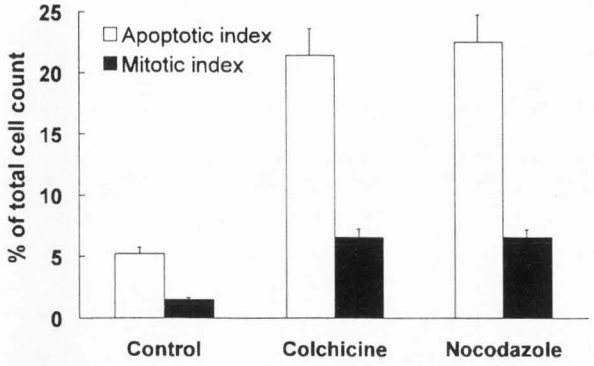

Figure 1. Apoptotic and mitotic indexes (mean values ± standard deviation, SD) of thymocytes incubated for 4 hours without drugs (Control), with 2 μM colchicine, or 2.5 μM nocodazole.

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confirms that pharmacologically effective doses of colchicine and nocodazole were used.

Both microtubule disrupting drugs raised AI in thymocyte cultures. This effect is seen already after one hour; a steady increase in the number of apoptotic cells occurs during the following three hours, exceeding 20% at 4 hours (Fig. 3). In untreated control cultures, around 5% of cells are apoptotic after 4 hours (Figs. 1 and 3). A small proportion of thymocytes (2-4%) in culture showed signs of necrosis; the proportion is independent of the treatment.

Electron microscopic analysis confirmed the normal appearance of the large majority of thymocytes incubated for 4 hours without drugs (Figs. 4a and 5c). After colchicine or nocodazole treatment, ultrastructural features of apoptosis became apparent in a number of cells (Figs. 4b and 5b). These include nuclear alterations, such as characteristic peripheral chromatin condensation and dispersion of nucleoli, but also cytoplasm condensation, and subplasmalemmal dilatation of the endoplasmic reticulum (ER), while mitochondria were initially intact (Fig. 6a). Necrotic stigmata, notably disruption of plasmalemma and swelling of mitochondria, not identified by light microscopy, were evident in a small number of cells which were exposed to colchicine or nocodazole for 4 hours (Fig. 6b). Based on nuclear appearance we consider these changes as a secondary necrosis of apoptotic cells.

No necrotic signs, such as marked swelling of mitochondria and disruption of the plasmalemma, were found in cells arrested in prometaphase. Nevertheless, some degenerative changes occurred during longer incubation times (4 hours), and were expressed by dilatation of ER, condensation of the cytoplasm and compaction of chromosomes (Fig. 7). The absence of the nuclear envelope and the irregular surface of chromatin masses (con-

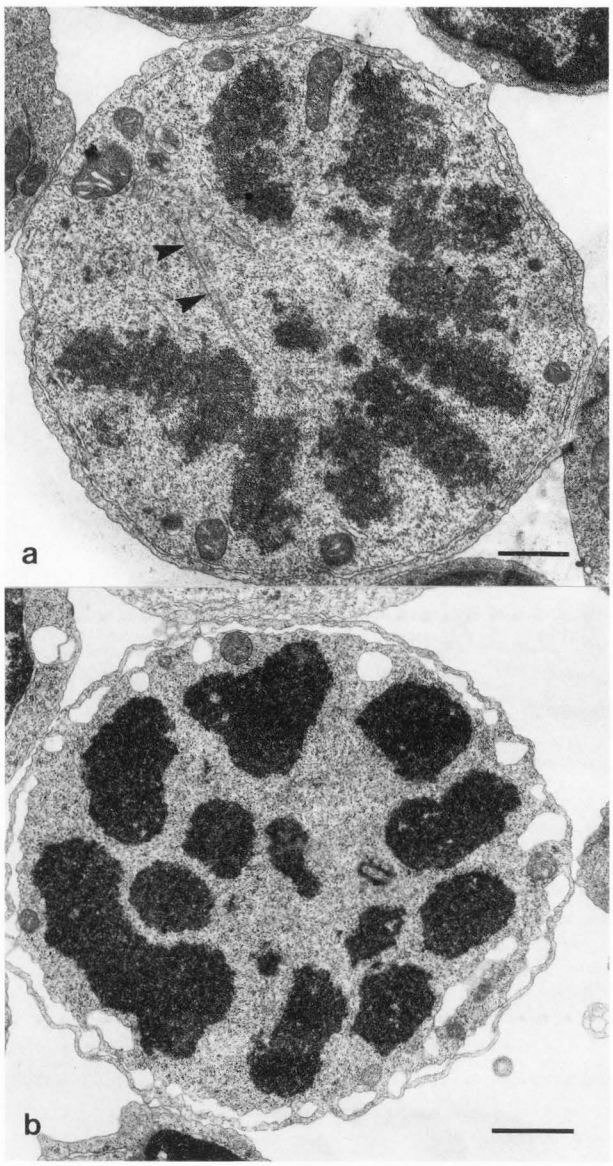

Figure 2. (a) EM appearance of mitotic thymocyte (control). Note spindle microtubules (arrowheads). (b) Mitotically arrested thymocyte showing absence of spindle microtubules; 1 hour after incubation with 2 μM colchicine). Bar = 1 μm.

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densed and clumped chromosomes) are suggestive of their relation to the mitotically arrested cells. Finally, large cytoplasmic splits produced by fusion of dilated cisternae of ER occurred and fragmentation of cells into smaller membrane-bound bodies containing parts of chromosomes can be also seen (Fig. 7b).

DNA electrophoresis

Morphological results showing that apoptosis can be triggered by microtubule disrupting drugs, are confirmed

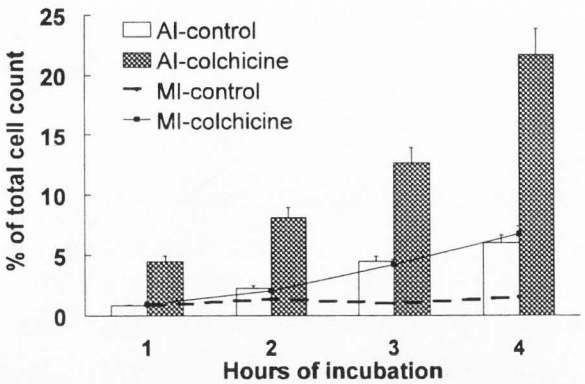

Figure 3. Time-course of AI and MI (mean values ± SD) incubated with or without 2 μM of colchicine for 1 to 4 hours.

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by DNA electrophoresis. A characteristic "ladder" pattern of internucleosomal DNA cleavage, a sign of endogenous endonuclease activity, is seen 4 hours after application of either colchicine or nocodazole (Fig. 5a). A similar pattern is observed after thymocyte exposure to dexamethasone, a drug known to induce apoptosis in cultured thymocytes (data not shown).

Effects of cycloheximide, EGTA and zinc

Zinc, EGTA, and cycloheximide by themselves, at the concentration used, did not have any noticeable effect on thymocytes incubated for up to 4 hours.

Inhibition of protein synthesis by 50 μM cycloheximide abrogates induction of apoptosis by colchicine or nocodazole (Fig. 8). Also, cycloheximide lowers MI to control values.

Chelation of Ca^{2+} in the incubation medium (nominal concentration 2.0 mM) by 5 mM EGTA markedly reduces apoptosis. EGTA does not have an effect on MI increase caused by colchicine or nocodazole (Fig. 8).

Inclusion of endonuclease-inhibiting concentrations of zinc (5 mM) in the incubation medium prevents appearance of the full spectrum of apoptotic alterations by colchicine or nocodazole. Nevertheless, early morphological signs of apoptosis occur, such as peripheral chromatin condensation (Fig. 9).

Discussion

This study demonstrates that MDD, colchicine and nocodazole trigger apoptotic cell death in isolated murine thymocytes. The process occurs within an hour of exposure of cells to the drugs, and requires the presence of Ca^{2+} and protein synthesis.

Assessment of apoptosis in this study is based on previously set morphological criteria [31, 33, 34]. Electrophoresis demonstrated DNA fragmentation, a biochemical marker of apoptosis; an endogenous calcium-

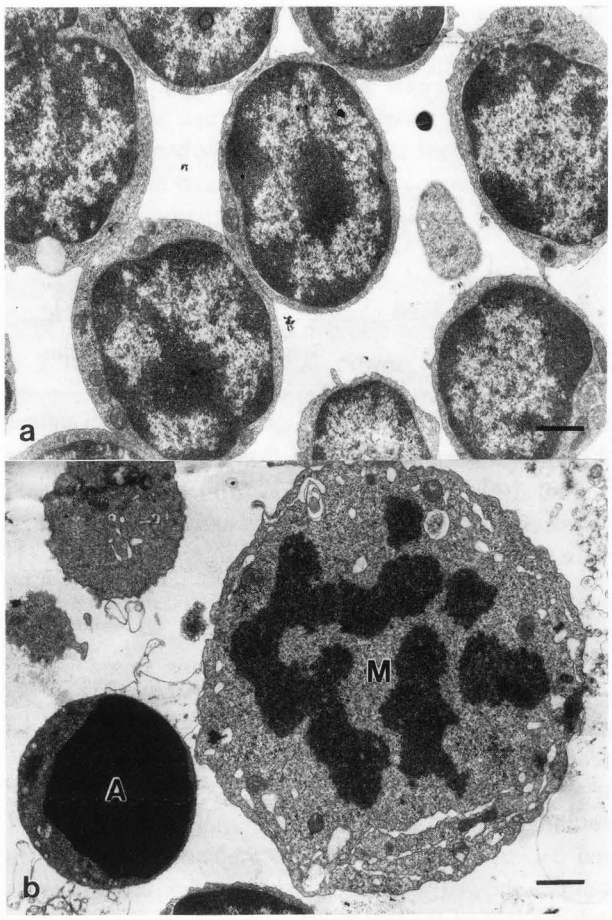

Figure 4. Electron micrographs of thymocytes. **(a)** Ultrastructural appearance of control thymocytes after 4 hours of incubation in medium without drugs. **(b)** Apoptotic thymocyte (A) and mitotically arrested (M); 4 hours after application of colchicine. Bar = 1 μm

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and magnesium-dependant endonuclease is presumably responsible for the DNA cleavage [32]. Activity of this nuclease is inhibited by zinc [8], which prevented induction of DNA fragmentation by colchicine and nocodazole in this study (data not shown). DNA fragmentation was also observed by treatment of HL-60 [17], BM 13674 lymphoma cell line [26] and rat hepatocytes [29] by MDD, thus, indicating that apoptosis is a major form of cell death induced by these drugs.

The effects of colchicine and nocodazole on the microtubular network, i.e., dissolution of microtubules (Fig. 2), is probably the cause of apoptosis in thymocytes exposed to the drugs *in vitro*. Although colchicine affects nucleoside transport [21] and protein synthesis [10], these effects are probably not responsible for the observed induction of apoptosis. Nocodazole, a specific microtubular poison without "side-effects" [11], induced

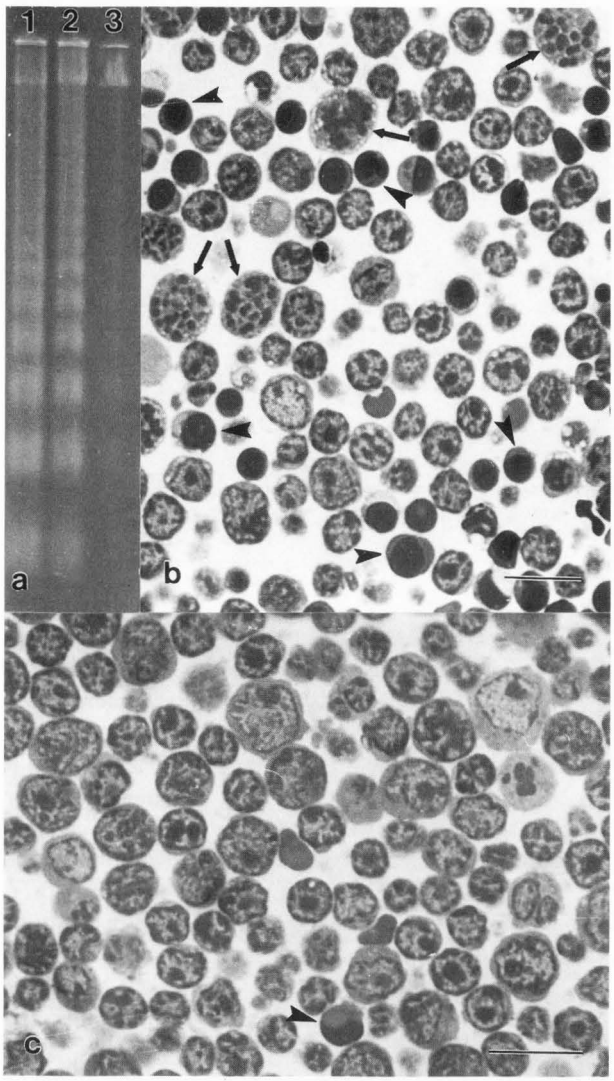

Figure 5. (**a**) DNA electrophoresis in 1.5% agarose. DNA was extracted from thymocytes incubated for 4 hours with 2 μM colchicine (Lane 1), 2.5 μM nocodazole (Lane 2), or without drugs (Lane 3). (**b**) Toluidine blue stained 1 μm section of thymocytes incubated 4 hours with colchicine. Note numerous apoptotic thymocytes (arrowheads) and mitotically arrested cells (arrows). (**c**) Thymocytes incubated 4 hours without MDD (control). Bar = 10 μm.

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apoptosis to an extent comparable to colchicine (Figs. 1 and 8). Induction of apoptosis was clearly separate from the effects of these drugs on mitotic blockade. Increase in the number of apoptotic cells is detected very early, one hour after exposure to drugs. It is unlikely that during this short period mitotic block could precede apoptosis. Moreover, electron microscopy showed no changes in the cells arrested in prometaphase during the

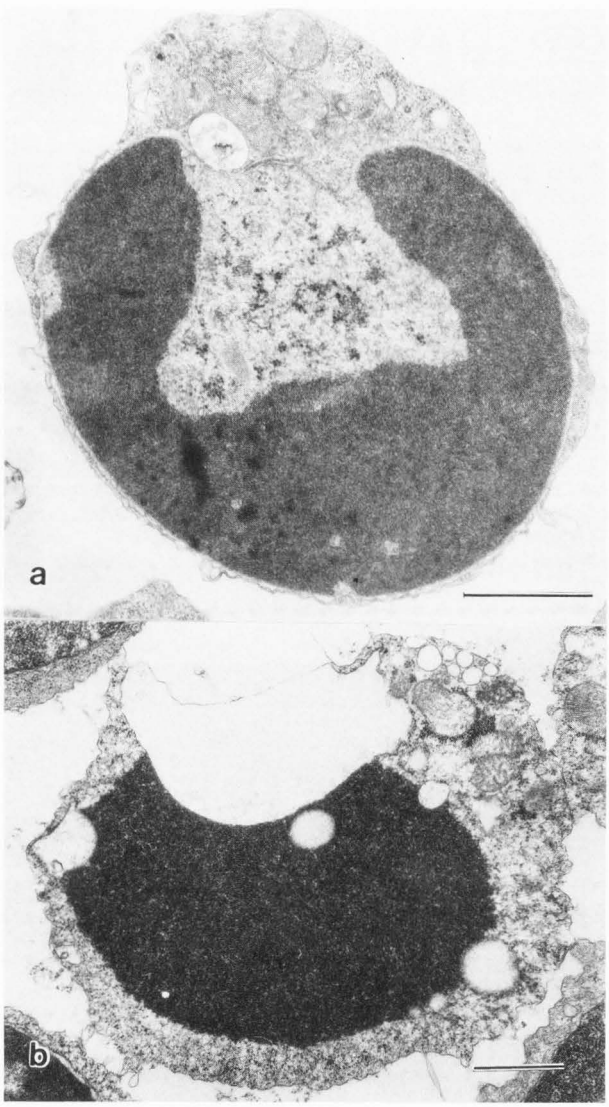

Figure 6. Characteristic ultrastructural features of apoptosis (**a**) and secondary necrosis (**b**) induced with colchicine. Note nuclear and cytoplasmic alterations (see text). Bar = 1 μm.

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first three hours of incubation. Our findings are in agreement with the study of Martin and Cotter [17], who distinguished mitotic from apoptotic effects of microtubule disrupting drugs. They observed necrosis of HL-60 cells arrested in prometaphase only 24 hours after drug exposure. We detected a small percentage of necrotic cells (2-4%) after 4 hours of incubation, but they were still showing some apoptotic features (Fig. 6b), and this we considered a secondary necrosis of apoptotic thymocytes. It has been shown that cells undergoing apoptosis in culture, where they usually escape phagocytosis, eventually degenerate by necrosis [33].

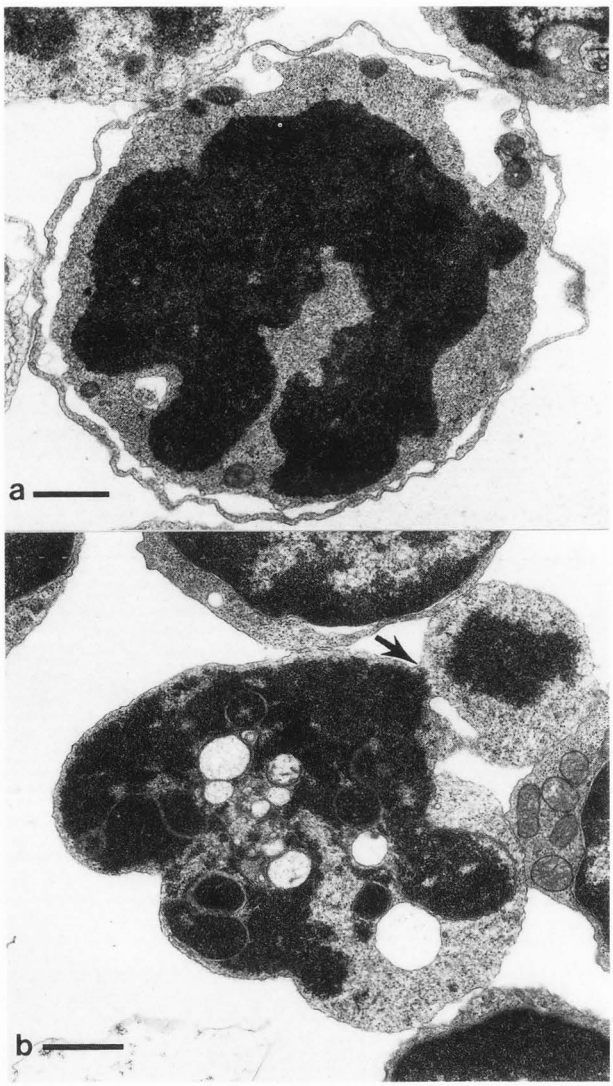

Figure 7. Ultrastructural appearance of mitotically arrested cells, 4 hours after incubation with colchicine (**a**) and nocodazole (**b**). Note condensation of the cytoplasm, dilatation of ER and clumping of chromosomes into coarse dense masses, and fragmentation of cell into smaller membrane-bound fragments (arrow). Bar = 1 μm.

The link coupling depolymerization of microtubules to apoptosis is not known. Early sustained increase of Ca^{2+} was observed in thymocytes undergoing apoptosis induced by various agents [3, 19, 20]. On the other hand, the rearrangement of microtubules was postulated during apoptosis induced by calcium-ionophores [17]. Our results show that chelation of extracellular Ca^{2+} by EGTA significantly reduces the number of apoptotic thymocytes induced by colchicine or nocodazole. It is well known that Ca^{2+} inhibits microtubule assembly,

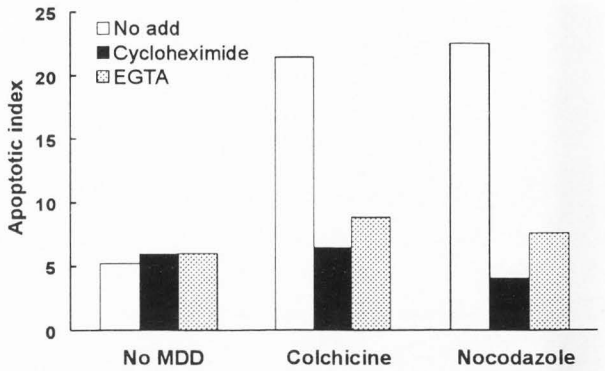

Figure 8. Effects of 50 μM cycloheximide, and 5 mM EGTA on induction of apoptosis by 2 μM colchicine or 2.5 μM nocodazole (4 hours of incubation). No add: no addition of cycloheximide, or EGTA.

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probably by binding to calmodulin which leads to the activation of Ca^{2+}-calmodulin dependent protein kinase, and that phosphorylation of microtubule associated proteins reduces their ability to stimulate microtubule polymerization [16]. It should be noted, however, that EGTA did not influence the rate of mitotic arrest, and it can be argued that calcium influx is not necessary for the action of drugs on microtubules. Thus, the calcium influx might be a consequence of the disruption of the cytoskeleton, and an initiating factor for apoptosis, which seems to be calcium-dependent in the majority of cells [22].

Inhibition of protein synthesis prevents apoptosis induced by various agents in thymocytes and other cell lines [8, 19, 35, 36]. Cycloheximide, a protein synthesis inhibitor, prevents apoptosis induced by colchicine or nocodazole (Fig. 8). Besides, it also lowers the mitotic index. It remains to be elucidated whether blocking of protein synthesis by cycloheximide prevents occurrence of apoptosis and lowers mitotic index by affecting synthesis of proteins involved in the process of programmed cell death, or by interfering with production of proteins necessary for cell cycle propagation, thus preventing cells from reaching the "apoptosis-prone" stage.

Our findings on the cycloheximide effects on apoptosis induced by colchicine and nocodazole is somewhat at variance with the report of Martin *et al.* [18], who showed that blockade of macromolecular synthesis enhances the apoptotic effect of microtubule disrupting drugs in HL60 cells after 24 hours of exposure. The considerably longer incubation time in that study may account partially for the differences in the drug effect. Cycloheximide alone, at a concentration of 10^{-5} M, has no substantial toxic/apoptotic effects upon thymocytes, T-cell lines, or on leukaemic lymphocytes during short incubation periods comparable to the periods employed

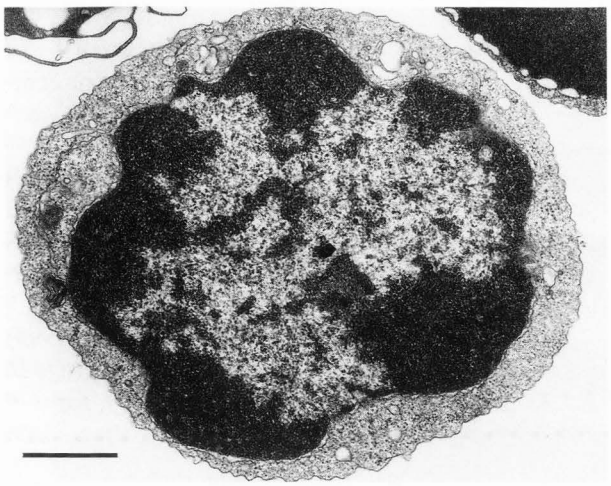

Figure 9. Ultrastructural characteristics of thymocyte incubated for 4 hours with colchicine in the presence of 5 mM zinc-sulphate. Observe early chromatin condensation and alteration of the nuclear shape. Bar = 1 μm.

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in this study [8, 30, 35]. We did not observe toxic effects of cycloheximide during the 4 hours of incubation. In a recent study, cycloheximide was ineffective in preventing MDD induced apoptosis in BM 13674 cells [26], which could be explained by activation of a pre-existing endonuclease which seems to be sufficient for apoptosis in some leukemic cells [9]. It appears that whether protein synthesis is necessary for apoptosis to occur is determined not only by the inducing agent, but also by the cell type.

Although it has been shown that HL-60 cells, arrested in prometaphase by MDD, die by necrosis [17], the present study, and the report by Harmon *et al.* [13], demonstrate apoptotic features in cells arrested in mitosis for several hours,such as, condensation of the cytoplasm, dilatation of ER and fragmentation into membrane-bound bodies containing chromosomal parts. It is a matter of speculation as to whether or not the same cellular machinery involved in apoptosis of interphase thymocytes is responsible for morphological changes in mitotically MDD-arrested cells. It should be noted that even in the absence of nuclei, apoptosis can occur [15].

In conclusion, two microtubule disrupting drugs, colchicine and nocodazole, induce apoptosis in murine thymocytes. Although the exact nature of the link between cytoskeleton disruption and apoptosis remains to be elucidated, the effect is specific and not related to the arrest of cells in mitosis. Nevertheless, these cells degenerate and die by a process morphologically similar to apoptosis. Apoptosis induced by colchicine and nocodazole requires the presence of calcium ions, unimpaired protein synthesis, and it is associated with internucleosomal DNA fragmentation. As these requirements have been described for other agents inducing apoptosis, it seems likely that colchicine and nocodazole activate the same, presumably common, pathway of programmed cell death.

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Discussion with Reviewers

T.M. Seed: The observation and comment that cycloheximide blocks MDD-induced apoptosis in normal mu-

rine thymocytes, but has the opposite effect in other cell systems (notably certain leukemic cell types) raises the possibility that there are different initiating events for the apoptosis pathway in normal versus cancer cells. Please comment.
Authors: The RNA and protein synthesis in apoptosis is dependent not only on the nature of the inducing agent, but even more so on the cell type [43]. Cells that do not require new protein synthesis to undergo apoptosis may repress apoptosis effector molecules that are already present in the cell. Such cells can be considered already "primed" for apoptosis by having undergone the early steps at some stage of their differentiation. It is possible that in certain leukemic cell types such "priming" might occur during malignant transformation.

E. Falcieri: Do you know if early morphological signs of apoptosis found in the presence of zinc progress, with time, towards a clear apoptosis? In any case, can you interpret these data?
Authors: It is becoming more clear that formation of larger DNA fragments is responsible for early morphological nuclear changes at the onset of apoptosis [41]. Zinc inhibits Ca^{2+}-Mg^{2+} dependent endonuclease that later cleaves DNA into oligo- and mononucleosomal size fragments seen as DNA "ladder" on agarose gels. Thus, in our study, zinc inhibited this type of DNA fragmentation and concomitant morphological changes. Based on our data alone, we cannot predict whether these early changes progress in time, or whether such thymocytes die by secondary necrosis, although the occurrence of apoptotic bodies have been found in the absence of extensive DNA fragmentation [38].

E. Falcieri: Among the morphological criteria for identifying apoptosis, could the nuclear pore distribution [39] provide new information in your model also?
Authors: It is clear that any additional morphological feature, that is always present in apoptosis, would be of a great help in providing more accurate criteria for identification of apoptosis. Thus, the specific nuclear pore distribution, which you have described [39], would be very interesting to examine during MDD induced apoptosis, especially in cells undergoing secondary necrosis.

K.M. Anderson: Do you distinguish between apoptosis and programmed cell death?
Authors: Apoptosis is only one form of the programmed cell death, and it has been shown that the programmed cell death can also have other morphological appearances [37]. Furthermore, apoptosis can occur in different pathological conditions, and can be induced by various treatments, so it is not always "programmed cell death" in the strict sense. However, to the best of our knowledge, the only form of the programmed cell death found in thymocytes is apoptosis.

K.M. Anderson: Which kinases are activated when cytosol Ca^{2+} increases? Can this "explain" any of the events you observed? How about phosphatases?
Authors: Increase in cytosol Ca^{2+} can activate several kinases, including Ca^{2+}-calmodulin dependent kinase, an enzyme with broad substrate specificity. By phosphorylating proteins associated with microtubules, it can mediate disassembly of microtubules. Also, calmodulin, which is an activator of adenyl cyclase in some cells, may act in thymocytes by causing an increase in intracellular cAMP, activation of protein kinase A (PKA) and subsequent apoptosis [42]. Moreover, PKA is associated with microtubules, and phosphorylation of the regulatory subunit of PKA releases the catalytic subunit and presumably allows it to phosphorylate other targets [16]. Microtubule disruption by MDD could also "release" PKA, which in turn could be more accessible to activation. Although protein phosphatases have opposite effects from protein kinases, calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase, has been implicated in the propagation of apoptosis, because immunosuppressants cyclosporin A and FK506 can block apoptosis in some model systems by inhibiting this phosphatase [44]. Thus, it could be also involved in Ca^{2+}-triggered apoptosis.

Reviewer V: Murine thymocytes generally die in culture as evidenced by substantially fewer cells at 24 and 48 hours. Why do you suggest that thymocytes may need to reach a stage of the cell cycle where they "are apoptosis-prone" as opposed to apoptosis induction at virtually any stage of the cell cycle? Evidence from other systems suggest that apoptosis can occur at many different stages of the cell cycle.
P.L. Olive: The apoptotic index in untreated thymocytes can be quite high after several hours *in vitro*. Is there is a possibility that these microtubule disrupting drugs are simply accelerating a process which would occur anyway in these cells?
Authors: After prolonged incubation, the majority of thymocytes die by apoptosis. But, in our experiments, we used short incubation time where MDD induced apoptosis in 20% of thymocytes, compared to 5% in the control. It is possible to speculate that majority of thymocytes are "primed" to die by apoptosis and that MDD accelerates this effect, but we have no data to substantiate this assumption. We believe that MDD induction of apoptosis in thymocytes could be different from their eventual apoptosis in the absence of "rescue factors" from thymic microenvironment, and that MDD effects might be aimed at the proliferating pool of

thymocytes. Although apoptosis can occur during any stage of the cell cycle, some drugs preferentially induce apoptosis in one stage of the cell cycle [40].

P.L. Olive: Can the authors tell us approximately what proportion of the thymocytes are in cycle?
Authors: Based only on our results, it is difficult to tell precisely the proportion of cycling thymocytes, although judging by the mitotic index it could be around 7% (Fig. 3). This is in agreement with previous study using thymidine incorporation [45], but values of even 20% have been reported [40].

P.L. Olive: Do authors feel that there is always a clear distinction, morphologically, between mitotic, apoptotic and necrotic cells?
Authors: It seems that established criteria are not always decisive in some cases. Certainly, this is true for secondary necrosis, when both apoptotic and necrotic features are present. It is even more complicated to distinguish between degenerative mitosis and apoptosis, so additional and more precise criteria are needed.

P.L. Olive: Could EGTA, in these experiments, act, even in part, by inhibiting the apoptotic endonuclease?
Authors: The apoptotic endonuclease is activated by Ca^{2+}, and EGTA would thus prevent its activation by preventing Ca^{2+} entry into the cell. The direct effect of EGTA on the apoptotic endonuclease is somewhat less likely, as cell membranes are not permeable for EGTA.

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